Temperature Suppresses Decay on Apple Fruit by Affecting *Penicillium solitum* Conidial Germination, Mycelial Growth and Polygalacturonase Activity

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Abstract: *Penicillium solitum* causes blue mold on apples during storage which results in economic losses. Information pertaining to growth and decay caused by this pathogen is important for developing disease control strategies. Therefore, we evaluated the effect of temperature on decay caused by *P. solitum* in apples, fungal growth in culture and quantitatively and qualitatively assayed polygalacturonase (PG) activity. Decay was evident only on apple fruit incubated at 20°C following 21 days incubation. However, decay developed when apples were moved from low temperatures (0, 5 and 10) to 20°C, which shows that the inoculum was viable following cold exposure. *P. solitum* viability at low temperature was confirmed as decay developed on inoculated apples following incubation for 3 months at 0°C. Lower temperatures reduced conidial germination and mycelial growth on PDA, which may explain the delay in decay development in inoculated fruit which was stored at 0, 5 and 10°C. Crude extracts from *P. solitum* decayed apple fruit tissue possessed detectable PG activity using the qualitative plate assay at 20°C but not at 0, 5, or 10°C. However, a quantitative method for determining *in vitro* PG activity using purified enzyme was observed at 0, 5, 10 and 20°C and decreased with temperature. Although, *P. solitum* is able to decay apple fruit following prolonged cold storage, low temperature is beneficial as it delays lesion development by affecting various factors of pathogen virulence.

Key words: Temperature, postharvest, decay, blue mold, *P. solitum*, polygalacturonase

INTRODUCTION

Losses attributed to postharvest decay of apples have been estimated to exceed $4.4 million per year in the United States (Rosenberger, 1997). *Penicillium solitum* and *P. expansum* are closely related species that cause blue mold of apples. Infection occurs primarily via wounds in the epidermis but also can occur through the sinus between the calyx and core cavity (Spotts *et al.*, 1999). They differ in virulence on pome fruit, with *P. expansum* being the more aggressive pathogen, but both cause significant economic losses (Rosenberger, 1990, 1997). Quality fruit are available year-round due to refrigeration and controlled atmosphere storage. Proper temperature management is critical to postharvest disease control and any other treatments are supplemental to refrigeration (Adaskaveg *et al.*, 2002). Low temperatures also have been shown to reduce spore germination and or mycelial growth in other fungal plant pathogens (i.e., *Rhizopus sexualis*, *R. stolonifer*, *Mucor piriformis*, *Botrytis allii*, *Monolinia laxa* and *M. fructicola*)

(Dennis and Blijham, 1980; Phillips, 1982; Tamm and Fluckiger, 1993; Tian and Bertolini, 1995, 1999).

The plant cell wall is the primary barrier to invasion by phytopathogens (Walton, 1994). It contains pectin, cellulose, hemicelluloses and proteins. Pectin is complexed with hemicellulose and provides cell wall integrity, tissue organization and adds texture to fruits and vegetables (Pilnik and Voragen, 1991). Pectin is composed of long chains of D-galacturonic acid polymers linked by α-1,4-glycosidic bonds. Polygalacturonases (PG) (EC 3.2.1.15) hydrolyze polygalacturonic acid via a single displacement mechanism which cleaves the α-1,4-glycosidic bond (Niture, 2008). PG activity coincides with diseases characterized by tissue maceration and soft rot. Their role in pathogen virulence has been shown via analysis of PG gene mutants (Bateman and Millar, 1966; Bateman and Basham, 1976; Shieh *et al.*, 1997; Wu *et al.*, 2008).

The objectives of this study were to determine the impact of temperature on *P. solitum* apple decay, fungal growth in culture and PG activity *in vitro*. The selected

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temperatures reflect those occurring in storage and handling such as: long term (0°C), fresh cut (5°C), abusive (10°C) and grocery store shelf (20°C).

MATERIALS AND METHODS

The *P. solitum* isolate was obtained from Dr. Robert Spotts (Oregon State University) and was routinely cultured on Potato Dextrose Agar (PDA). 'Golden Delicious' apple fruits were harvested from a commercial orchard (Bear Mountain) in Gardners, Pennsylvania at Rice Fruit Company in 2008. All chemicals used in this work were purchased from Sigma Chemical Co. (St. Louis, MI), unless otherwise specified.

Golden Delicious apple fruit were inoculated with 50 μL of a conidial suspension at 10^3 conidia mL^{-1} as described by Jurick *et al.* (2009). Control fruit were inoculated with sterile water only. Inoculated and control fruit were incubated at 0, 5, 10, or 20°C±0.1°C and lesion diameter was measured after 3, 7, 14 and 21 days. Then, apple fruit with no discernable lesion were placed at 20°C±0.1°C and monitored until decay was evident. Five apples composed a replicate, there were 2 replicates per treatment and the experiment was repeated. Two replicates of 15 *P. solitum* inoculated apples and 5 control fruit, were incubated at 0°C for 3 months and decay was evaluated.

Skin from the lesion or inoculation point was removed aseptically with a scalpel and approximately 2.5 g of cortical tissue from 3 apples were removed from the center of the inoculated area with a No. 10 cork borer. Tissue was then pooled and homogenized with an equal volume of 100 mM MES + 150 mM NaCl pH 6.0 using a battery powered hand blender. The homogenate was filtered through cheesecloth, squeezed and the filtrate was placed in a labeled 50 mL conical tube. Approximately 50 μL of the filtrate from decayed apples incubated at 0, 5, 10 or 20°C and a pectinase solution from *Aspergillus niger* (positive control) were assayed for polygalacturonase (PG) activity using the PG plate method as previously described (Jurick *et al.*, 2009). The PG plate test was used to qualitatively assay PG activity from decayed tissue because background reducing sugars from the apple do not interfere with detection of enzyme activity as with the quantitative *in vitro* reducing sugar assay. Three plates per replicate were used to assay PG activity and the experiment was repeated.

Conidia were harvested from actively sporulating cultures of *P. solitum*, grown on PDA for 2 weeks. Petri plates (60 mm), each containing 10 mL of PDA, were inoculated with 250 μL of conidial suspension (2×10^7 conidia mL^{-1}) of *P. solitum*. Plates were incubated at 0, 5, 10, or 20±0.1°C and observed after 1, 3 and

7 days. For each time point, five plates were evaluated by haphazardly observing 100 conidia per plate and percent germination was calculated.

P. solitum was cultured on PDA and plugs (5 mm in diameter) were cut from the edge of actively growing 2-week-old cultures and placed in the center of 90 mm Petri plates containing PDA. Plates were then incubated at 0, 5, 10, or 20±0.1°C and the colony diameter was recorded after 3, 7, 14 and 21 days. Five replicates were used for each treatment and the experiment was conducted twice. Mycelial growth rates (mm day^{-1}) at different temperatures were calculated based on a 7 day interval (14-21 days post inoculation) representing the most vigorous phase of fungal growth.

Polygalacturonase from apple fruit tissue decayed at 20°C by *P. solitum* was purified according to a method described previously (Jurick *et al.*, 2009). Activity tests of the purified enzyme were carried out at 0, 5, 10 or 20°C for 20 min according to method of Gross (1982). Control reactions were executed by adding borate-borax buffer with 2-cyanoacetamide prior to the addition of substrate buffer and were incubated with each sample to determine background levels of reducing sugars.

All data were analyzed as general linear mixed models or regression models with Proc Mixed or Proc Reg (Sas Institute). The assumptions of the models were checked and when necessary the variance grouping technique was used to correct variance heterogeneity. Mean comparisons were done with Sidak adjusted p-values so that the experiment-wise error was 0.05. Germinated conidia were classified into three categories <25, 25-85 and >85% and the counts were analyzed as doubly stratified contingency tables using exact Jonckheere-Terpstra tests (StatXact, Cytel Software Corp.) to determine if the distribution of germinating conidia differed over temperature. Diameters of fungal colonies were analyzed as linear regressions with time as the independent variable.

RESULTS AND DISCUSSION

Decay developed on apples at 20°C, but was not evident on apples inoculated with *P. solitum* at 0, 5, or 10°C after 21 days incubation (Fig. 1a). Nevertheless, lesions developed within 14 days on all fruit following transfer from low temperatures (0, 5 and 10°C) to 20°C, indicating that the fungus remained viable. This result is supported by the observation that after 3 months incubation at 0°C, *P. solitum* inoculated fruit developed decay with a 73% incidence. Unlike *P. solitum*, some postharvest pathogens (i.e., *Rhizopus stolonifer* and *Aspergillus niger*) are sensitive to low temperatures
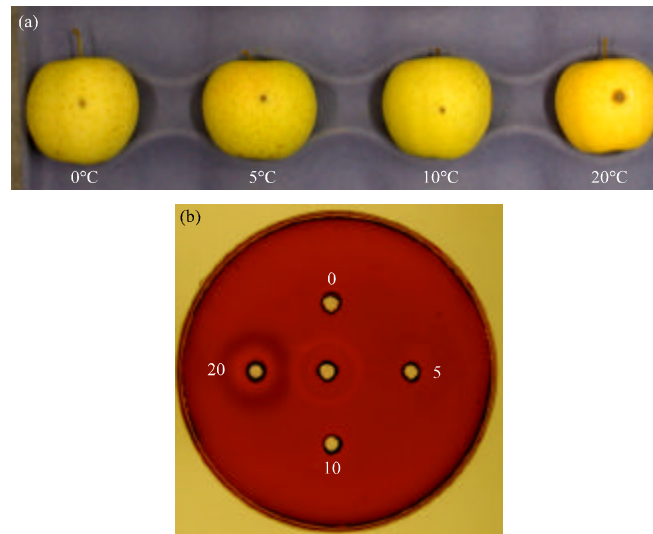
Fig. 1: (a) Golden delicious apple fruit with lesions caused by *Penicillium solitum* incubated at various temperatures for 21 days. (b) PG plate assay of crude extracts from *P. solitum*-decayed apple fruit at 0, 5, 10, 20°C and positive control in the center well. Clearing zones indicate PG activity on ruthenium red stained background. The experiment was repeated and results from a representative experiment are shown

which are biocidal after several days (Adaskaveg *et al.*, 2002; Dennis and Blijham, 1980). Therefore, fruit contaminated with *P. solitum* may serve as a source of inoculum with the potential to initiate the disease cycle. Temperature also affected PG activity in crude extracts of decayed apple fruit (Fig. 1b). PG activity occurred concomitantly with fungal decay and agrees with previous data which reported that PG is involved in *P. solitum*-mediated decay of apple fruit (Jurick *et al.*, 2009). However, the effect of other fungal cell wall degrading enzymes (i.e., cellulase, protease, etc.) during decay cannot be excluded.

To elucidate the delaying effect of temperature on *P. solitum* apple decay, we investigated the impact of temperature on *P. solitum* conidial germination and mycelial growth *in vitro*. Following 24 h incubation, conidia germinated at only 10 and 20°C on PDA. Nevertheless, conidial germination was observed at 5°C and 0°C after 3 and 7 days, respectively (Table 1). Delayed conidial germination *in vitro* in response to low temperature has previously been shown for *P. expansum* but not *P. solitum* (Brooks and Cooley, 1917). Mycelial growth rate and overall colony diameter were reduced as incubation temperature decreased (Fig. 2a, b). Similar results have been obtained for mycelial growth, conidial germination and infection of garlic bulbs with *Botrytis allii* (Tian and Bertolini, 1995). Low incubation temperatures also have been shown to retard growth, spore production, formation and germination of *Monilinia laxa* (Tamm and Fluckiger, 1993; Tian and Bertolini, 1999)
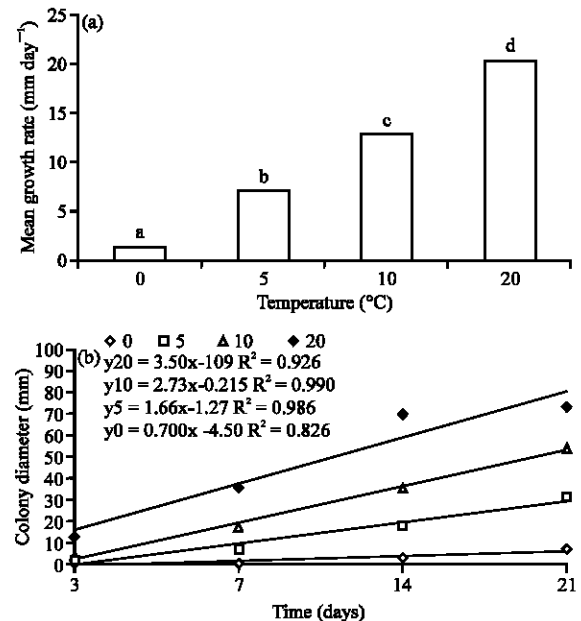

Fig. 2: (a) Mean mycelial growth rate of *Penicillium solitum* at various temperatures. Data represent the means from 2 experiments. Mean growth rates with different letters are statistically significant at the 0.05 level using Sidak adjusted p-values. (b) Linear regressions of *P. solitum* mycelial growth on potato dextrose agar at various temperatures over time. The slopes of the lines were different (p<0.05) and data represent the means from 2 experiments

Table 1: Germination of *Penicillium solitum* conidia over time at 0, 5, 10 and 20°C

Time (days)	Temperature (°C)			
	0	5	10	20
1	[x]0c	0c	5.1b	99.7a
3	0	79.4	n.c	n.c
7	41.3	[y]n.c	n.c	n.c

[x]Means with different letters are statistically different p<0.001 according to the Jonckheere-Terpstra test and data represent the means from 2 experiments. [y]Not counted as plates were overgrown
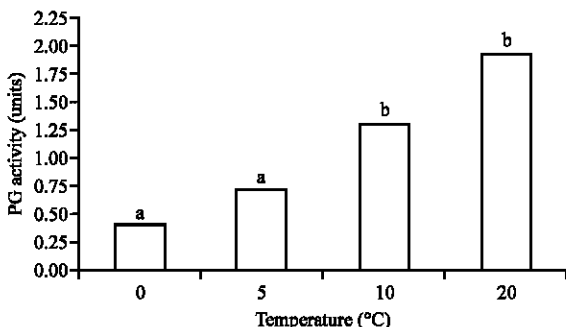

Fig. 3: Effect of temperature on the *in vitro* enzyme activity of purified *Penicillium solitum* polygalacturonase. Data represent the means from 2 experiments and bars with different letters are significantly different p = 0.05 according to the Sidak adjusted p-values test

and conidial size in *M. fructicola* (Phillips, 1982). Brooks and Cooley (1917) demonstrated that the mycelium of *P. expansum* was able to grow on PDA at low temperatures including 0°C. However, a weakly virulent pathogen like *P. solitum* was not previously tested for growth at 0°C, but has been shown to grow at 5°C with a similar rate shown in this study (Pitt *et al.*, 1991).

Based on these findings, we hypothesized that the lack of decay was due to delayed fungal growth at low temperature which also impacted PG production and activity and thus prevented decay. Therefore, to determine the potential of PG enzyme activity at temperatures where no decay was observed *in vivo*, we assayed PG activity using purified enzyme from *P. solitum* decayed apple fruit *in vitro*. Purified PG was active at 0, 5, 10 and 20°C and activity decreased with temperature (Fig. 3). Therefore, since fungal growth and PG activity occur at 0°C, decay will develop over extended periods of storage as shown in this study (i.e., 3 months). Data presented have also shown that *P. solitum* can remain viable during storage at temperatures as low as 0°C. Therefore, cold treatments should remain constant during long-term storage which will delay decay and inhibit subsequent infections when fruit are removed from storage and placed on store shelves.

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